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Twentieth day of October 2004

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PROVISIONAL SPECIFICATION

Applicant:

BIONOMICS LIMITED

A.C.N. 075 582 740

Invention Title:

A DIAGNOSTIC METHOD FOR EPILEPSY

The invention is described in the following statement:

A DIAGNOSTIC METHOD FOR EPILEPSY

Background Art

Epilepsies constitute a diverse collection of brain disorders that affect about 3% of the population at some time in their lives (Annegers, 1996). An epileptic seizure can be defined as an episodic change in behaviour caused by the disordered firing of populations of neurons in the central nervous system. This results in varying degrees of involuntary muscle contraction and often a loss of consciousness. Epilepsy syndromes have been classified into more than 40 distinct types based upon characteristic symptoms, types of seizure, cause, age of onset and EEG patterns (Commission on Classification and Terminology of the International League Against Epilepsy, 1989). However the single feature that is common to all syndromes is the persistent increase in neuronal excitability that is both occasionally and unpredictably expressed as a seizure.

A genetic contribution to the aetiology of epilepsy has been estimated to be present in approximately 40% of affected individuals (Gardiner, 2000). As epileptic seizures may be the end-point of a number of molecular aberrations that ultimately disturb neuronal synchrony, the genetic basis for epilepsy is likely to be heterogeneous. There are over 200 Mendelian diseases which include epilepsy as part of the phenotype. In these diseases, seizures are symptomatic of underlying neurological involvement such as disturbances in brain structure or function. In contrast, there are also a number of "pure" epilepsy syndromes in which epilepsy is the sole manifestation in the affected individuals. These are termed idiopathic and account for over 60% of all epilepsy cases.

Idiopathic epilepsies have been further divided into partial and generalized sub-types. Partial (focal or local) epileptic fits arise from localized cortical discharges, so that only certain groups of muscles are

involved and consciousness may be retained (Sutton, 1990). However, in generalized epilepsy, EEG discharge shows no focus such that all subcortical regions of the brain are involved. Although the observation that generalized epilepsies are frequently inherited is understandable, the mechanism by which genetic defects, presumably expressed constitutively in the brain, give rise to partial seizures is less clear. In neonates and infants, probably because brain myelination is incomplete, the distinction between generalized and partial epilepsies is less clear from clinical and neurobiological standpoints.

Epilepsies in the first year of life were previously viewed as largely due to acquired peri-natal factors. However, two benign autosomal dominant epilepsy syndromes are now well recognised in the first year of life. The first is benign familial neonatal seizures (BFNS) which usually presents around the third day of life and is characterised by tonic or clonic seizures. These seizures stop within a few weeks of age, with 5% of individuals having later febrile seizures and 11% later epilepsy (Plouin, 1994). Studies have shown that the genetic basis for this syndrome in a number of cases is due to mutations in the potassium channel genes KCNQ2 and KCNQ3.

The second is benign familial infantile seizures (BFIS) which presents between 4 and 8 months of age, with clusters of tonic or clonic partial or generalised seizures over a few days. Seizures usually resolve by around 1 year of age but it may be associated with paroxysmal dyskinesias in later childhood in some individuals. While no genes have been definitively identified to be causative of BFIS, linkage to chromosomes 19 and 16 have been reported (Szepetowski et al., 1997; Guipponi et al., 1997).

In 1983, prior to the recognition of BFIS, an American family was reported that had an intermediate variant of BFNS and BFIS, termed benign familial neonatal-infantile seizures (BFNIS), where seizure onset varied

from 2 days to 3.5 months (Kaplan and Lacey, 1983). Recently, genetic analysis of two BFNIS families lead to the identification of two mutations in the SCN2A gene that were responsible for the disorder.

The inventors have built on this study through the analysis of affected individuals from additional families with probable or possible BFNIS. This has lead to the identification of further missense mutations in SCN2A in 6 families that result in changes in evolutionary conserved amino acids. Both families recognised as probable BFNIS and 4/9 families recognised as possible BFNIS contained SCN2A mutations. This further emphasizes the importance of genetic factors in epilepsies of the neonatal and early infantile periods. Of 95 families with other forms of childhood epilepsy tested, none contained mutations in SCN2A.

Based on the determination that SCN2A mutations are highly specific for BFNIS the inventors have established a method for the diagnosis of BFNIS based on the identification of mutations (novel and known) in the SCN2A gene.

Disclosure of the Invention

In a first aspect of the present invention there is provided a method of identifying BFNIS-associated mutations in the SCN2A gene comprising the steps of selecting a system of assays comprising at least a first and final assay, said first assay being selected to provide a test for the existence of a BFNIS-associated mutation and said final assay being selected to provide a test to determine the nature of the BFNIS-associated mutation. There are a number of assay systems that may be selected to perform the diagnostic test and these are known to one skilled in the art. Examples are provided below.

In a second aspect of the present invention there is provided a method of testing patients for BFNIS-associated mutations in the SCN2A gene comprising the steps of:

(1) selecting a system of assays comprising at least a first and final assay, said first assay being selected to provide a test for the existence of a BFNIS-associated mutation and said final assay being selected to provide a test to determine the nature of the BFNIS-associated mutation;

(2) performing said first assay; and, if the results indicate the existence of a BFNIS-associated mutation,

(3) performing said second assay.

In one embodiment an assay system employed may be the analysis of SCN2A DNA from a patient sample in comparison to wild-type SCN2A DNA. Genomic DNA may be used for the diagnostic analysis and may be obtained from body cells, such as those present in the blood or cheek, tissue biopsy, surgical specimen, or autopsy material. The DNA may be isolated and used directly for the diagnostic assays or may be amplified by the polymerase chain reaction (PCR) prior to analysis. Similarly, RNA or cDNA may also be used, with or without PCR amplification.

In one embodiment, an assay to analyse heteroduplex formation may be employed. By mixing denatured wild-type SCN2A DNA with a DNA sample from a patient, any sequence variations in the SCN2A sequence between the two samples will lead to the formation of a mixed population of heteroduplexes and homoduplexes during reannealing of the DNA. Analysis of this mixed population can be achieved through the use of such techniques as high performance liquid chromatography (HPLC) which are performed under partially denaturing temperatures. In this manner, heteroduplexes will elute from the HPLC column earlier than the homoduplexes because of their reduced melting temperature.

In a further embodiment, a DNA hybridisation assay may be employed. These may consist of probe-based assays specific for the SCN2A gene. In an SCN2A exon-specific assay, the probe-based assay will utilize at least one probe which specifically and selectively hybridises to an exon of the SCN2A gene in its wild-type form. Thus, the lack of formation of a duplex nucleic acid hybrid containing the nucleic acid probe is indicative of the presence of the mutation in the SCN2A gene. Because of the high specificity of probe-based tests, any negative result is highly indicative of the presence of the BFNIS-associated mutation and further investigational assays should be employed to determine the nature of the mutation.

The SCN2A exon-specific assay approach could also be adapted to identify previously determined SCN2A mutations responsible for BFNIS. In this aspect, a probe which specifically and selectively hybridises with the SCN2A gene in its mutant form is used. In this case the formation of a duplex nucleic acid hybrid containing the nucleic acid probe is indicative of the presence of the mutation in the SCN2A gene. In each variation of the exon-specific assay approach, it is important to take into account known polymorphisms in the SCN2A gene that are not associated with BFNIS. A secondary assay such as DNA sequencing should subsequently be employed to ensure that any suspected mutations are not known polymorphisms.

The exon-specific probes used for these assays may be derived from: (1) PCR amplification of each exon of the SCN2A gene using intron specific primers flanking each exon; (2) cDNA probes specific for each exon; or (3) a series of oligonucleotides that collectively represent an SCN2A exon.

In a further embodiment, patient samples may be subject to electrophoretic-based assays. For example electrophoretic assays that determine SCN2A fragment length differences may be employed. Fragments of each

patient's genomic DNA are amplified with SCN2A gene intron-specific primers. The amplified regions of the SCN2A gene therefore include the exon of interest, the splice site junction at the exon/intron boundaries, and a short portion of intron at either end of the amplification product. The amplification products may be run on an electrophoresis size-separation gel and the lengths of the amplified fragments are compared to known and expected standard lengths from the wild-type gene to determine if an insertion or deletion mutation is found in the patient sample. An SCN2A gene mutation may be diagnosed if such length variations are identified. This procedure can advantageously be used in a "multiplexed" format, in which primers for a plurality of exons (generally from 2 to 8) are co-amplified, and evaluated simultaneously on a single electrophoretic gel. This is made possible by careful selection of the primers for each exon. The amplified fragments spanning each exon are designed to be of different sizes and therefore distinguishable on an electrophoresis/size separation gel. The use of this technique has the advantage of detecting both normal and mutant alleles in heterozygous individuals. Furthermore, through the use of multiplexing it can be very cost effective.

In a further approach, diagnostic electrophoretic assays for the detection of previously identified SCN2A mutations may utilise PCR primers which bind specifically to mutated exons of the SCN2A gene. In this case, product will only be observed in the electrophoresis gel if hybridization of the primer occurred. Thus, the appearance of amplification product is an indicator of the presence of the mutation, while the length of the amplification product may indicate the presence of additional mutations.

Additional electrophoretic assays may be employed. These may include the single-stranded conformational polymorphism (SSCP) procedure. As mentioned above, fragments of each patient's genomic DNA are PCR amplified

with SCN2A gene intron-specific primers such that individual exons of the SCN2A gene are amplified and may be analysed individually. Exon-specific PCR products are then subjected to electrophoresis on non-denaturing polyacrylamide gels such that DNA fragments migrate through the gel based on their conformation as dictated by their sequence composition. SCN2A exon-specific fragments that vary in sequence from wild-type SCN2A sequence will have a different secondary structure conformation and therefore migrate differently through the gel. Aberrantly migrating PCR products in patient samples are indicative of a mutation in the SCN2A exon and should be analysed further in secondary assays such as DNA sequencing to determine the nature of the mutation.

In a further embodiment, enzymatic based assays may be used in diagnostic applications. Such assays include the use of S1 nuclease, ribonuclease, T4 endonuclease VII, MutS, Cleavase and MutY.

When a diagnostic assay is to be based upon the SCN2A protein, a variety of approaches are possible. For example, diagnosis can be achieved by monitoring differences in the electrophoretic mobility of normal SCN2A protein and SCN2A protein isolated from a test sample. Such an approach will be particularly useful in identifying mutants in which charge substitutions are present, or in which insertions, deletions or substitutions have resulted in a significant change in the electrophoretic migration of the resultant protein. Alternatively, diagnosis may be based upon differences in the proteolytic cleavage patterns of normal and mutant proteins, differences in molar ratios of the various amino acid residues, or by functional assays demonstrating altered function of the gene products.

Further assays that are based on the SCN2A protein include immunoassays. Immunoassays for the SCN2A gene product are not currently known. However, immunoassay is included in the selection of assays because the procedures

for raising antibodies against specific gene products are well described in the literature, for example in U.S. Pat. Nos. 4,172,124 and 4,474,893 which are incorporated herein by reference. Antibodies are normally raised which bind to portions of the gene product away from common mutation sites such that the same antibody binds to both mutant and normal protein. Preferred antibodies for use in this invention are monoclonal antibodies because of their improved predictability and specificity. It will be appreciated, however, that essentially any antibody which possesses the desired high level of specificity can be used, and that optimization to achieve high sensitivity is not required.

For the diagnostic detection of mutations in SCN2A involved in BFNIS, antibodies raised against the defective gene product is preferable. Antibodies are added to a portion of the patient sample under conditions where an immunological reaction can occur, and the sample is then evaluated to see if such a reaction has occurred. The specific method for carrying out this evaluation is not critical and may include enzyme-linked immunosorbant assays (ELISA), described in U.S. Pat. No. 4,016,043, which is incorporated herein by reference; fluorescent enzyme immunoassay (FEIA or ELFA), which is similar to ELISA, except that a fluoregenic enzyme substrate such as 4-methylumbelliferyl-beta-galactoside is used instead of a chromogenic substrate, and radioinmunoassay (RIA).

The most definitive diagnostic assay that may be employed is DNA sequencing. Comparison of the SCN2A DNA wild-type sequence with the SCN2A sequence of a test patient provides both high specificity and high sensitivity. The general methodology employed involves amplifying (for example with PCR) the DNA fragments of interest from patient DNA; combining the amplified DNA with a sequencing primer which may be the same as or different from the amplification primers; extending the sequencing primer in the presence of normal nucleotide (A,

C, G, and T) and a chain-terminating nucleotide, such as a dideoxynucleotide, which prevents further extension of the primer once incorporated; and analyzing the product for the length of the extended fragments obtained. While such methods, which are based on the original dideoxysequencing method disclosed by Sanger et al., 1977 are useful in the present invention, the final assay is not limited to such methods. For example, other methods for determining the sequence of the gene of interest, or a portion thereof, may also be employed. Alternative methods include those described by Maxam and Gilbert (1977) and variations of the dideoxy method and methods which do not rely on chain-terminating nucleotides at all such as that disclosed in U.S. Pat. No. 4,971,903, which is incorporated herein by reference. Any sequence differences (other than benign polymorphisms) in SCN2A exons of a test patient when compared to that of the wild-type SCN2A sequence indicate a potential disease-causing mutation.

In specific embodiments of the invention, there is provided a method for testing patients for BFNIS-associated mutations in the SCN2A gene comprising the steps of:

a) quantitatively amplifying at least one exon of the SCN2A gene from a body sample of each patient to produce amplified fragments;

b) comparing the properties of the amplified fragments to standard values based upon the fragments produced by amplification of the same exon in a non-mutant SCN2A gene; and

c) determining the nucleic acid sequence of each exon identified in b) that has different properties in the patient compared to the corresponding non-mutant SCN2A exon.

In further specific embodiments there is provided a method for testing patients for BFNIS-associated mutations in the SCN2A gene comprising the steps of:

a) quantitatively amplifying, from a body sample of each patient at least one exon of the SCN2A gene using primers complementary to intron regions flanking each amplified exon;

b) comparing the length of the amplification products for each amplified exon to the length of the amplification products obtained when a wild-type SCN2A gene is amplified using the same primers, whereby differences in length between an amplified sample exon and the corresponding amplified wild-type exon reflect the occurrence of a truncating mutation in the sample SCN2A gene; and

c) determining the nucleic acid sequence of each exon identified in b) to contain a truncating mutation.

In even further specific embodiments there is provided a method for testing patients for BFNIS-associated mutations in the SCN2A gene comprising the steps of:

a) quantitatively amplifying, from a body sample of each patient at least one exon of the SCN2A gene using primers complementary to intron regions flanking each amplified exon;

b) hybridising the fragments from a) with fragments produced by amplification of the same exon in a non-mutant SCN2A gene;

c) determining the nucleic acid sequence of each patient exon identified in b) that either does not hybridise to corresponding wild-type fragments or forms a mismatched heteroduplex.

Throughout this specification and the claims, the words "comprise", "comprises" and "comprising" are used in a non-exclusive sense, except where the context requires otherwise.

It will be apparent to the person skilled in the art that while the invention has been described in some detail for the purposes of clarity and understanding, various modifications and alterations to the embodiments and

methods described herein may be made without departing from the scope of the inventive concept disclosed in this specification.

Modes for Performing the Invention

Any combination of assay systems described above may be employed for the diagnosis of SCN2A mutations responsible for BFNIS. Provided below is an example of an assay combination.

Example 1: SCN2A electrophoretic assay using SSCP

DNA from a test patient may be obtained in a number of ways. The most common approach is to obtain DNA from blood samples taken from the patient, however DNA may also be obtained using less invasive approaches such as from cheek cell swabs.

For this specific example DNA was extracted from collected blood using the QIAamp DNA Blood Maxi kit (Qiagen) according to manufacturers specifications or through procedures adapted from Wyman and White (1980). Stock DNA samples were kept at a concentration of 1 ug/ul.

Once DNA is obtained, PCR amplification of individual exons of the SCN2A gene may be employed prior to analysis by SSCP. Table 1 provides a list of primers that may be employed to analyse each exon of the SCN2A gene.

In this specific example, primers used for SSCP were labelled at their 5′ end with HEX and typical PCR reactions were performed in a total volume of 10 µl. All PCR reactions contained 67 mM Tris-HCl (pH 8.8); 16.5 mM $(NH_4)_2SO_4$; 6.5 µM EDTA; 1.5 mM $MgCl_2$; 200 µM each dNTP; 10% DMSO; 0.17 mg/ml BSA; 10 mM β-mercaptoethanol; 5 µg/ml each primer and 100 U/ml *Taq* DNA polymerase. PCR reactions were typically performed using 10 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds followed by 25 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. A final extension reaction for 10 minutes at 72°C followed.

Ten to twenty µl of loading dye comprising 50% (v/v) formamide, 12.5 mM EDTA and 0.02% (w/v) bromophenol blue were added to completed reactions which were subsequently run on non-denaturing 4% polyacrylamide gels with a cross-linking ratio of 35:1 (acrylamide:bis-acrylamide) and containing 2% glycerol. Gel thickness was 100µm, width 168mm and length 160mm. Gels were run at 1200 volts and approximately 20mA, at 18°C and analysed on the GelScan 2000 system (Corbett Research, Australia) according to manufacturers specifications.

Example 2: DNA sequencing assay

PCR products from the SSCP analysis that showed a conformational change may be subject to secondary assays such as DNA sequencing to determine the nature of the change. In the example provided here, this first involved re-amplification of the amplicon displaying a band-shift from the relevant individual (primers used in this instance did not contain 5' HEX labels) followed by purification of the PCR amplified templates for sequencing using QiaQuick PCR preps (Qiagen) based on manufacturers procedures. The primers used to sequence the purified amplicons were identical to those used for the initial amplification step. For each sequencing reaction, 25 ng of primer and 100 ng of purified PCR template were used. The BigDye sequencing kit (ABI) was used for all sequencing reactions according to the manufacturers specifications. The products were run on an ABI 377 Sequencer and analysed using the EditView program.

A comparison of the DNA sequence obtained from the patient sample can then be made directly to that of the wild-type SCN2A sequence in order to detect the DNA alteration that lead to the conformational change detected by SSCP. If the DNA change is not a known polymorphism in the SCN2A gene, it is likely that it may be a disease causing mutation essentially providing a diagnosis that can be investigated further through the analysis of additional family members.

TABLE 1

Primer Sequences Used for Mutation Analysis of SCN2A

Exon	Forward Primer	Reverse Primer	Size(bp)
5'UTR	ACAGGAAGTTAGGTGTGGTC	GAGAAGCATCACAGAG	206
1a	TGCTGTATCTCAGTGCTCAG	TCATCATCCTCATCCTTGCG	281
1b	GCTAAGAGACCCAAAC	TAGGCAGTGAAGGCAACTTG	201
2	GGCACTATTTTACAGGGC	CATAACATTGCCAACCACAG	325
3	TGGTGAAGGCATGGTAGT	ATTGAGGAGGTCTCAAGGTG	239
4	ACCAACCTGGAAGTGTCT	ATAGTATAGGCTCCCACCAG	300
5	AGGCCCCTTATATCTCCAAC	TAGCAACAAGGCTTCTGCAC	244
5n	GATGAAAGACCAAGGAAGAC	TGGAGATATAAGGGGCCTAG	200
6a	TTCCAGGACAAGCTCATG	GGAAGAATTATCTGGAGGCCA	249
6b	TTGTTCATGGGCAACCTACG	GTCTAAGTCACTTGATTCAC	271
7	GTGAGCTTTGCCACCTAAAC	TGAGAGTCACCGTGAAGTAG	280
8	ACCAATTAGCAGACTTGCCG	CTACAGCAATTCTCTTGAG	264
9	CTCAAGAGAATTGCTGTAG	AGGACCGTATGCTTGTTCAC	326
10a	TTCCACATACTTTGCGCCCTTC	GCTGTCTTCAGATTCCGA	235
10b	CAGAAAGAACAGTCTGGAG	CTCTGAAAGCATTGTGCCA	256
11a	CCACATGTCCAATGAC	CACGAACAGAGAGTCTCTTC	296
11b	TGATGAGCACAGCACCTTTG	CACCAGTCACAACTCTCTTC	281
12	CTTTGGGCTTTGCTGCTTTC	AAGTAACTGTGACGCAGGAC	222
13a	CCTCCAGCAGATTAACCCAT	CAGGTCAACAAATGGGTCCA	268
13b	ACACCTTGTCAACCTGGTTG	GATGTCAAGATATACATGGCC	258
14	CCCGTGTTTCAAGAGTATTTGCTC	GCTTATGAACACTCCCAG	252
15a	GCAGAGCATTAACACTGTTC	AGCGTGGGAGTTCACAATCA	241
15b	GCATGCAGCTCTTTGGTAAG	CCCTTCAGTTGAACACAC	299
16a	CCTGTTTTTCCTGCTGTGTTTC	GCCACTAGTAGTTCCATTTCCGTC	336
16b	GACAGCTGTATTTCCAACC	AACAGGAAGGAAACACGC	346
17	CTGACCTTTACCAAAGCGGA	GAGGATACTCAAGACCAC	318
18	TGAATCTCCCACCAACAC	GAGTGGATCATGCATCACCT	252
19	CTTAGGCACCTGATAAGAGC	AAAGCAGCAAAGTGCAGC	302
20	CATTGCATAGAGCAAGGC	GGTACAAAGTGTCAGTCTGCTCTC	263
21a	TTTCCTTCTCATCCTGTGCC	CTGGCAGTTTGATTGCTCTC	240
21b	AGCGTGGTCAACAACTACAG	GCCATTCTAACAGGTGGA	217
22	GCCCCAAAAGTGAATAC	GCGCCAATTTCCCTCTAACTAGAC	224
23	GGGCCCAGAGATTAAAACATGC	CAGAGCAAGGATGAAG	272
24	GAATGAAATGTGGGAGCC	TTCGGGCTGTGAAACGGTTA	266
25a	TTACCTCAGCTCTCCAATCACTGG	TGGTCATCGGTTTCCACCAT	292
25b	TCATCTGCCTTAACATGGTC	GGGAGTTTGGGATGAATG	311
26a	GTACCTAACTGTCCTGTTCAC	TAAACAACGCAGGAAGGGAC	270
26b	CACGCTGCTCTTTGCTTTGA	GATCTTTGTCAGGGTCACAG	269
26c	GGATGGATTGCTAGCACCTA	TCGCATCGGGATCAAACTTC	281
26d	AGCCTCTGAGTGAGGATGAC	TCCATCTGTATTCGAAGGGC	277
26e	GTGAGAGTGGAGAGATGGAT	TATCATACGAGGGTGGAGAC	330
26f	AACCGATATGACGCCTTCCA	GGTCTCTGTCTTGTTATAGGC	288

Note: Primer sequences are listed 5' to 3'. Due to the large size of exons 1, 6, 10, 11, 13, 15, 16, 21, 25 and 26, the exons were split into two or more overlapping amplicons. The neonatally expressed exon 5 is represented as exon 5n.

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